

Succession of bacteria attached to evergreen oak leaf surfaces

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Received September 27, 1993; accepted December 9, 1993.

Abstract

Heterotrophic attached bacteria were isolated from evergreen oak leaf surfaces (*Quercus ilex* L.) on both phylloplane and litter at each season during one year. Few epiphytic bacteria were recovered from the phylloplane (18%) in comparison with the litter. The data derived from the 324 strains were subjected to cluster analysis. The taxonomic structure displayed 44 phenetic groups. The bacterial communities on leaf surfaces belonged mainly to two genera: *Bacillus* (31%), present on both phylloplane and litter, and *Pseudomonas* (35%) present only on the litter. Some communities of *Bacillus* were able to degrade structural chemical components of the leaf, such as pectins or lignin monomers. The *Bacillus* isolates were generally more numerous in spring and in summer, *i.e.* during the warm seasons, whereas *Pseudomonas* predominated in autumn and winter. *In vitro* measurements of strains growth rates showed moreover that *Bacillus* strains grew better at 40°C than *Pseudomonas* strains. A climate-related factor, namely temperature, has therefore a great influence on the natural selection process of these communities. Another seasonal succession was observed, involving two genera: *Xanthomonas* predominant in winter and *Lactobacillus* in summer, as well as the *Enterobacteriaceae* family in autumn.

Keywords: Attached bacteria, leaves, phylloplane, litter, evergreen oak.

Successions bactériennes associées aux feuilles de chêne vert

Résumé

Les bactéries hétérotrophes associées aux feuilles de chêne vert (*Quercus ilex* L.) ont été isolées à partir de la phyllosphère et de la litière à chaque saison au cours d'une année. Un faible nombre de bactéries épiphytes proviennent de la phyllosphère (18%). Les données obtenues sur les 324 souches isolées sont analysées avec une méthode de classification hiérarchique. La structure taxonomique fait apparaître 44 phénons. Les communautés bactériennes des feuilles appartiennent principalement à deux genres : *Bacillus* (31 %) présent dans la phyllosphère et la litière, et *Pseudomonas* (35 %) présent seulement dans la litière. Certaines communautés de *Bacillus* peuvent dégrader des composés chimiques structuraux de la feuille, comme les pectines ou des monomères de la lignine. Les isolats de *Bacillus* sont plus nombreux pendant les saisons chaudes, ceux de *Pseudomonas* en automne et en hiver. Les taux de croissance mesurés *in vitro* montrent que les souches de *Bacillus* isolées se développent mieux à 40°C que celles de *Pseudomonas*. Un facteur climatique, la température, a donc une forte influence sur le processus de sélection de ces communautés. Une autre succession saisonnière existe entre le genre *Xanthomonas* dominant en hiver, *Lactobacillus* en été et la famille des *Enterobacteriaceae* en automne.

Mots-clés : Bactéries attachées, feuilles, phyllosphère, litière, chêne vert.

INTRODUCTION

Leaf degradation is initiated by microflora within the canopy, which contains a great number of microorganisms especially during senescence (Dickinson and Preece, 1976). The aerial leaf surfaces have been studied in various environments (Austin *et al.*, 1978; Ercolani, 1978). In litter, where leaf decomposition occurs, Jensen (1974) reported that microorganisms could degrade up to 80 % of the plant material. The microbial populations inhabiting the phylloplane and the litter include yeasts, filamentous fungi and bacteria (Dickinson, 1982). Most papers dealing with leaf surface focused on saprotrophic fungi (Dickinson and Preece, 1976). However, bacteria are generally reported to be the first invaders of young leaves (Jensen, 1974). Their relative abundance has often been underlined (Dickinson and Preece, 1976). Indeed, leaves can easily intercept and retain a varied air-driven bacterial flora (Leben and Whitmoyer, 1979) and under favorable temperature and moisture conditions, this resident population living on the leaf surface multiplies (Schneider and Grogan, 1977).

Many works concerning plant-surface bacteria focus on phytopathogenic bacteria because of their great economic importance (Yessad *et al.*, 1992). However, Billing (1987) has reported that it is not possible to distinguish between bacteria that multiply saprophytically and those that initiate disease. All the bacteria colonizing the leaf surface (epiphytic and pathogenic bacteria) participate in the biogeochemical cycles and should consequently be investigated. Moreover, bacterial number and metabolic activity are known to vary depending on the season and on the age of the leaves (Austin *et al.*, 1978). Taxonomic studies of bacterial communities from leaf surfaces are necessary to understand the physiology of plant-related bacteria and microbe-plant interactions. The further characterization of these bacteria which occur naturally on young, senescent and dead leaves is of crucial importance to determine their role in the decomposition of the litter.

The present work is part of a larger research project dealing with the degradation of evergreen oak leaf litter by microorganisms and microarthropods. Evergreen oak (*Quercus ilex* L.) forms characteristic forest climax populations which are widely distributed throughout the western Mediterranean area (Rapp, 1969). Leaves represent 48 % of the evergreen oak litter (Lossaint and Rapp, 1978). The biological activity through enzymatic action of microorganisms (Chabert *et al.*, 1984) and the role of microarthropods (Sadaka and Poinot-Balaguer, 1989) in the decay of the evergreen oak litter were previously investigated.

In this work, we attempt to determine the distribution of several heterotrophic bacteria attached to evergreen oak leaf surfaces on the tree and the litter at each season during one year. Bacterial

populations were recovered by an indirect method based on culture on nutrient agar medium after plating of washed leaf disks. The taxonomic diversity was investigated using numerical methods. We found that the dominant heterotrophic bacteria formed distinctive communities on phylloplane and litter. Changes in bacterial populations through the seasons were also detected.

MATERIAL AND METHODS

Sampling procedure

Evergreen oaks (*Quercus ilex* L.) grew in dense copses planted at "La Gardiole de Rians" (Var, France). The copses were 50 years old and formed a continuous cover. The climate and vegetation of this site were previously described (Barbero and Loisel, 1983). Leaves were collected in May (spring), September (summer), November 1986 (autumn), and February 1987 (winter). Four stages of leaves were considered:

- 1-year-old;
- 2-year-old;
- leaves typical of the litter upper layer (L_1);
- leaves still recognizable in spite of decay and constituting the litter underlayer (L_2).

At the time of each sampling, 300 leaves at each stage were collected from various trees and from the litter. Leaves of the same age-class were pooled in sterile flasks and processed promptly.

Bacterial strain isolation

Four disks, five millimeters in diameter, were aseptically cut out from each leaf. To remove unattached bacteria from leaf surfaces, 100 disks were placed in 150 ml sterile distilled water plus 0.1 % Tween 80. After 2 times 5 minutes washing by mechanical shaking, disks were rinsed in 10 changes of sterile distilled water 2 minutes per rinsing. Disks corresponding to 50 abaxial and 50 adaxial leaf surfaces were plated out (3 per plate) on Trypticase-Soy (TS) agar medium (bioMérieux, France) plus amphotericin B (0.01 g.l^{-1}).

A hundred disks shaken and rinsed as mentioned above were disinfected by shaking one minute in HgCl_2 solution (0.1 % w/v). Disks were then rinsed with sterile distilled water one time during 20 minutes and five times 2 minutes. The surface-disinfecting disks were plated on the TS medium.

After washing or disinfection treatment and 15 days incubation at 28°C , each colony developing around the plated disks was transferred onto new plates until purification. Strains were stored on Trypticase-Soy agar slants at 4°C .

Numerical valuation of unattached and attached bacteria on the leaves

Unattached bacteria present in the various washing and rinsing waters were counted. Attached bacteria that remained on leaf surfaces were removed by another washing treatment. They correspond to the bacteria present in the suspension water obtained after vigorous shaking of 100 washed disks in 60 ml sterile distilled water with a high-speed shaker at 2200 rpm (Bioblock Scientific, Illkirch, France) for 15 minutes. This time of shaking was selected because it allowed the greatest recovery of bacteria from the leaf surface. Suspensions-dilutions were plated on TS medium supplemented with amphotericin B (0.01 g.l^{-1}). Three Petri dishes were used for each dilution to determine bacterial numbers. Colony counts were averaged and converted to colony-forming units (CFU) per cm^2 .

Morphological, physiological and biochemical tests

Each strain was subjected to 98 phenotypic tests including 28 morphological and 70 biochemical, physiological or nutritional tests. The colonial morphology, the morphology of cells and their arrangement, the presence of endospores and the mobility were investigated from isolated colonies grown on TS agar for 1 to 2 days. The physiological and biochemical tests performed on each culture included Gram reaction (Cerny, 1976), oxidase test with Oxy-Swab technique (Biological Labs, Austin, Texas), the Hugh-Leifson test (1953), the pigment production and API tests. The API microtube tests named API 20E (bioMérieux, La Balme les Grottes, Montalieu-Vercieu, France) were used as standardized biochemical and physiological micromethods. API 20E strips were incubated during 48 hours at 28°C .

Nutritional tests

The nutritional characteristics were tested as growth on the following substrates: D-glucose, D-ribose, D-galactose, D-mannose, D-xylose, L-rhamnose, L-arabinose, cellobiose, D-gluconate, D-galacturonate, glycerol, succinate, acetate, propionate, L-phenylalanine, L-tryptophane, benzoate, *p*-hydroxybenzoate, 2,5-dihydroxybenzoate, 3,4-dihydroxybenzoate, *o*-phthalate, 2-hydroxyphenylacetate, 4-hydroxyphenylacetate, salicylate, 3-(4-hydroxyphenyl) propionate, syringic acid, *p*-coumaric acid, ferulic acid, vanillic acid, sinapic acid, protocatechuic aldehyde, veratraldehyde, vanillin, veratryl alcohol, coumaryl alcohol, sinapyl alcohol, coniferyl alcohol. The last three substrates were chosen because they are lignin monomers. These alcohols and related-acids occur in leaf degradation process. All the organic compounds mentioned were supplied as sole carbon source to a base medium (Stanier *et al.*, 1966) at

a concentration of 2 g.l^{-1} with glucides, 1 g.l^{-1} with acid by-products of glucides and amino-acids, 0.25 g.l^{-1} with fat acids and aromatic compounds. Growth was monitored on microtiter plates (ELISA) with a Multiskan Titertek unit.

Amylolytic and pectinolytic activities were tested by growing the strains on a solid mineral base medium (Deschamps and Lebeault, 1980). Degradation of 75 % methylated pectin was determined as described by Hankin and Anagnostakis (1975) after adding 5 g/l of citrus pectin (Sigma, Saint-Quentin Fallavier, France) to the base medium. Degradation of starch (Prolabo, Paris, France) was studied according to Buttiaux *et al.* (1962). Degradation of cellulose (Bolloré Technologie, Perpignan, France) and 22 % methylated apple pectin (Sanofi Bioindustrie, Baupré, France) were determined according to Pochon and Tardieux (1962).

Numerical analysis

The test results were subjected to a cluster analysis to determine the phenetic relationships among strains. Data were analysed on a Digital Equipment VAX/VMS computer. The strains were clustered on the basis of the minimum intragroup variance (Ward, 1963). Similarity (KHI_2 taxonomic distance) and clustering (variance algorithm) were calculated using the Jambu LSM CREDOC program (Benzecri, 1973). The genus or family were assigned to most of the clusters in line with the Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984; Sneath *et al.*, 1986).

Measurement of bacterial growth rates

Thirty six strains were chosen at random between isolates from leaves of various ages collected during the four different seasons. The growth of strains was measured on liquid Trypticase-Soy (bioMérieux, France) medium (dilution 10^{-1}) on microtiter plates in a spectrophotometer Metertech $\Sigma 960$ (Bioblock Scientific, Illkirch, France) at 405 nm. Inocula were made from a 20 hours preculture. Thirty two cupules were seeded for each strain to obtain mean values of adsorbance. The specific growth rates of strains were calculated after culture at three temperatures (5°C , 28°C , 40°C). The adsorbance was read every hour in the case of growth at 28°C or 40°C and every twelve hours in the case of growth at 5°C .

RESULTS

Numerical distribution of bacteria on the leaves

Attached bacteria amounted to $3.03 \cdot 10^4 \pm 0.22 \text{ CFU.cm}^{-2}$ on leaves of the tree and to $1.24 \cdot 10^6 \pm 0.31 \text{ CFU.cm}^{-2}$ on litter leaves. Unattached bacteria amounted respectively to $9.85 \cdot 10^2 \pm 1.6 \text{ CFU.cm}^{-2}$

and to $2.21 \cdot 10^4 \pm 0.04$ CFU.cm⁻². These bacteria only represented respectively percentages of 3.5 and 2.4 with regard to the bacteria still present on leaf surfaces after our washing treatment. It seems likely they correspond to the very weakly attached members of the phylloplane community. The total number of bacteria isolated from evergreen oak leaves with our plated leaf disks method amounted to 324. The recovery of isolates from leaves at each season was approximately constant. The number of strains obtained from the leaves was greater on the litter (265), particularly on L₂ leaves, than on the phylloplane (59). The bacterial strains isolated from surface-disinfecting disks amounted only to 5.5% of the 324 isolates and 61% of these strains were obtained from L₂ leaves. The disinfection with HgCl₂ did not affect the development of bacteria within the leaf structure, although the cells of the latter were partly destroyed (Racon *et al.*, 1988).

The phenotypic similarities among isolates are illustrated in a phenogram (*fig. 1*). Four main groups join at 0.05 taxonomic distance. At 0.008 taxonomic distance, the phenogram shows 44 clusters symbolized by triangles, each containing more than two strains. Of these clusters seven contain 10 or more isolates,

each of them representing only 26% of the bacterial population, twelve include 5 to 9 strains; 87 isolates distribute among the remaining 25 clusters, each of them containing 1 to 4 isolates.

Taxonomic distribution of bacteria

In the phenogram, groups 2 (97.6%) and 4 (87.5%) are more homogeneous than groups 1 (62.2%) and 3 (70.6%). These results and the main discriminating characters between the four groups are shown in Table 1. Weak percentages of isolates from group 4 were able to degrade the structural and reserve polymers of the leaves. Only 11 strains (3.4% of studied strains) were able to utilize aromatic compounds as sole carbon source (data not shown).

The taxonomic distribution of the clusters among the 4 groups is given in Table 2. The principle of the hierarchical classification which assigns the same weight to all the characters involves some heterogeneous clusters. These clusters include a small number of isolates corresponding to more than one genus but the difficulty of reliably identifying bacterial isolates from natural habitats (Sneath, 1989)

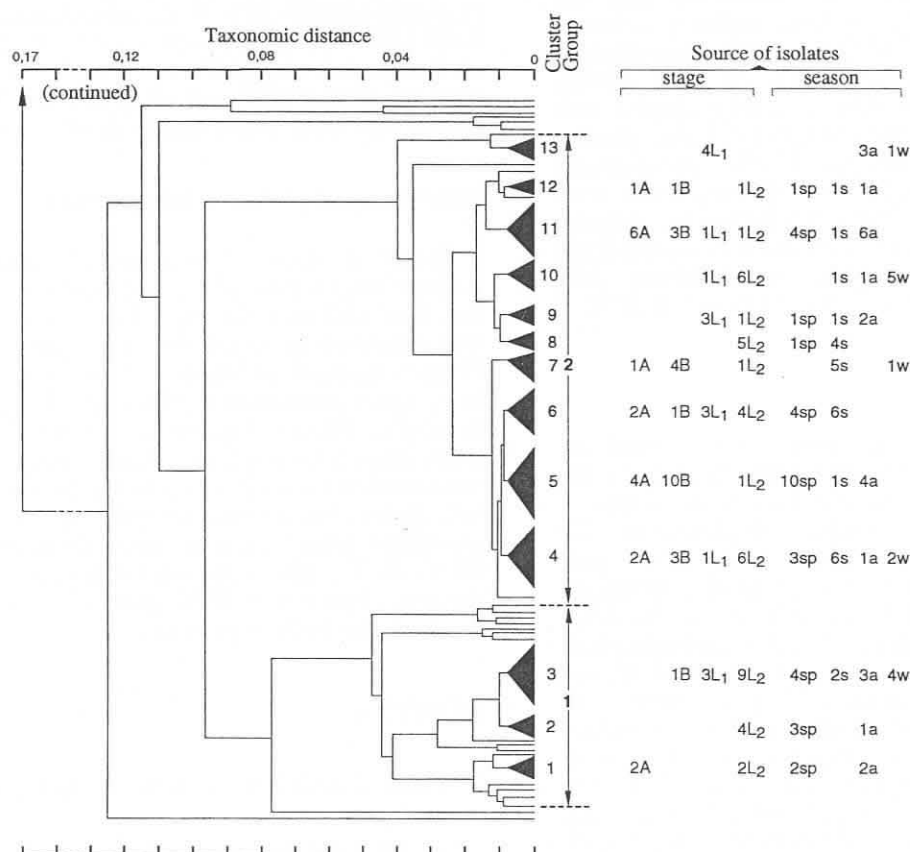


Figure 1. – (continued on next page). Phenogram of 324 isolates recovered from evergreen oak leaves of various stages at each of the 4 seasons during one year. The relationships shown are based on KHI₂ taxonomic distance and clustering on variance algorithm. The isolates are identified by their number, the stage of the leaves from which they were obtained (A = 1-year-old, B = 2-year-old, L₁ = litter upper layer, L₂ = litter underlayer) and the season when the leaves were collected (sp = spring, s = summer, a = autumn, w = winter).

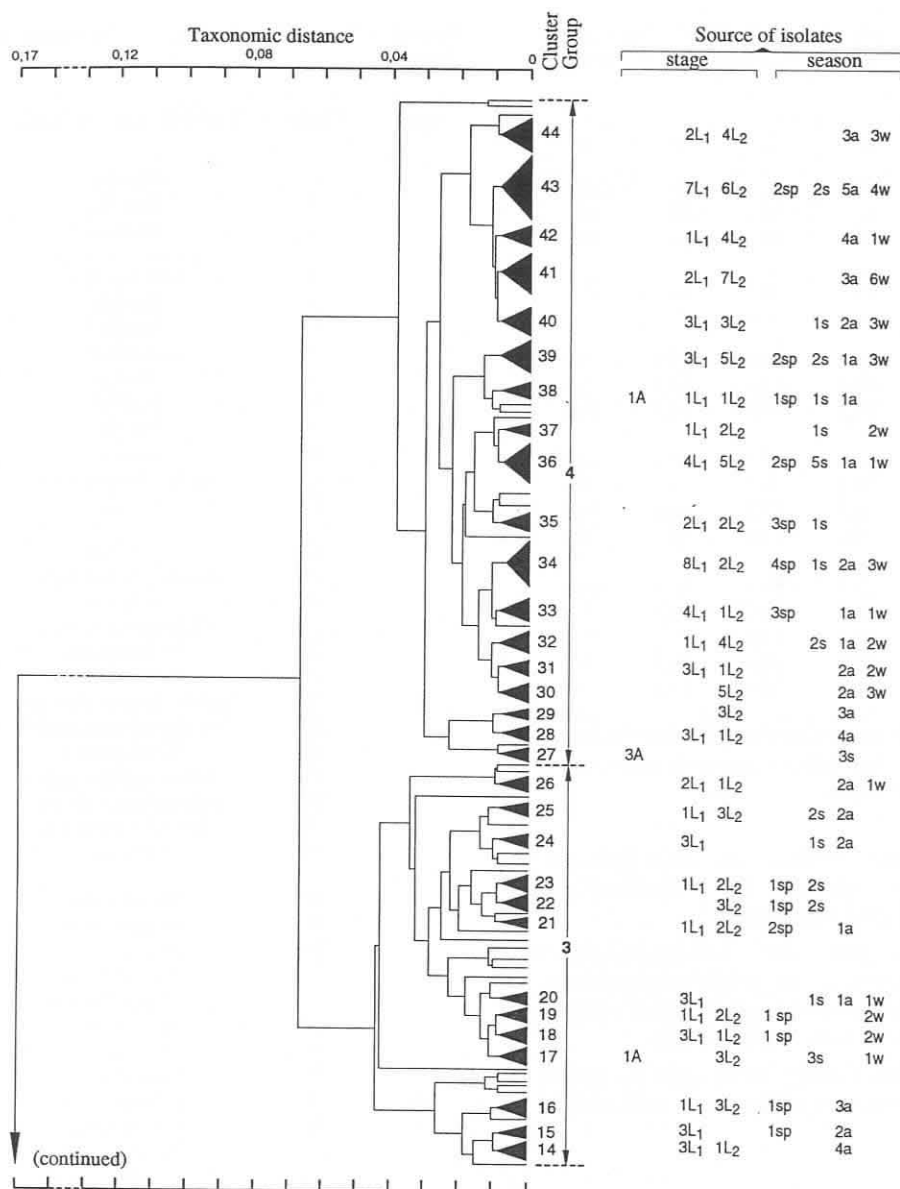


Figure 1. – (continued).

particularly from the aerial part of plants (Billing, 1976) is well known.

Most of the isolates belonged to two main genera *i.e.* *Pseudomonas* (35 %) and *Bacillus* (31 %) present in groups 4 and 2 respectively. The other strains distributed into the *Enterobacteriaceae* (8 %), particularly *Klebsiella* (1.5 %) and *Serratia* (2.2 %) and the following genera: *Lactobacillus* (9 %), *Xanthomonas* (3.7 %), *Arthrobacter* (1.9 %), *Kurthia* (1.5 %), *Micrococcus* (0.9 %), *Alcaligenes* (0.6 %), *Acinetobacter* (0.3 %), *Cellulomonas* (0.3 %), *Corynebacterium* (0.3 %) and *Curtobacterium* (0.3 %). In the phenogram, *Micrococcus* strains are not included into the 4 groups. Nine *Lactobacillus*, 7 *Bacillus*, 1 *Cellulomonas* and

1 *Pseudomonas* were isolated from surface-disinfected leaves. These genera were also found on non-disinfected leaves except for the *Cellulomonas* strain which was isolated only once from L₂ leaves.

Concerning the utilization of aromatic compounds, positive results were obtained with the following strains:

- one *Micrococcus* strain on vanillin;
- one *Corynebacterium* strain on 4-hydroxyphenylacetate;
- two *Arthrobacter* strains on 2- and 4-hydroxyphenylacetate, as well as on syringic and *p*-coumaric acids;

Table 1. – Distribution and characteristics of isolates in the 4 groups clustered at 0.05 taxonomic distance in the phenogram shown in figure 1.

Group	1	2	3	4
Number of strains	37	82	75	120
Strains (%) *	11	25	23	37
Number of clusters	3	10	13	18
Number of strains not included in a cluster	14	2	22	15
<i>Main discriminant characters</i>				
Gram-positive	89.2	95.2	25.3	3.3
Presence of spore	59.4	53.6	13.3	3.3
Mobility	75.7	97.6	89.3	99.2
Anaerobic fermentation				
of glucose	64.9	95.2	52.0	14.2
NO ₃ reduction	67.6	1.2	37.3	0.8
Oxidase test	8.1	17.9	36.0	89.2
β -Galactosidase (ONPG)	27.0	97.6	73.3	12.5
Hydrolysis of				
• gelatin	75.7	79.8	50.7	64.2
• starch	54.1	28.6	21.3	3.3
• cellulose	16.2	11.9	17.3	5.0
• 22% methylated pectin	18.9	94.0	21.3	8.3
• 75% methylated pectin	16.2	36.9	17.3	7.5

* % expressed with regard to the total number of isolated strains.

Note: The percentages of positive tests are expressed with regard to the total number of isolated strains.

– three *Pseudomonas* strains on 3-(4-hydroxyphenyl)propionate, on benzoate, on *p*-hydroxybenzoate, on sinapic acid and vanillin;

– four *Bacillus* strains on 3-(4-hydroxyphenyl)propionate, on benzoate, on *p*-hydroxybenzoate, as well as on coniferyl, coumaryl, sinapyl and veratryl alcohols, ferulic, syringic and sinapic acids.

Thus, *Bacillus* strains mainly were able to utilize aromatic structural monomers of lignin as sole carbon source.

Spatial succession of bacteria on the leaves

Ninety-three percent of the 2-year-old leaf isolates belonged to group 2, whereas the epiphytic bacteria isolated from the 1-year-old leaves were distributed among the 4 groups (fig. 1). The species diversity was consequently lower on older leaves. The distribution of the litter isolates depended on the level of sampling. The L₁ isolates predominated in group 2 (60% of this group isolates), whereas the L₂ isolates predominated in groups 1 (67%) and 2 (33%). The spatial fluctuations in the proportions of the various genera differed qualitatively and quantitatively (fig. 2). Colonization of the evergreen oak leaf surfaces began on the canopy since bacteria were isolated after the bud burst on the 1-year-old leaves. *Bacillus* was present on both phylloplane and litter. Ten % of the *Bacillus* strains were recovered from the phylloplane. *Pseudomonas* was present only in the litter.

Table 2. – Taxonomic distribution of the isolates among groups and clusters.

Group	Cluster	Probable genus or family	Number of isolates
1	1	<i>Kurthia</i>	4
	2	<i>Bacillus</i>	4
	3	<i>Bacillus</i>	13
2	4	<i>Lactobacillus</i>	12
	5	<i>Bacillus</i>	15
	6	<i>Bacillus</i>	10
	7	<i>Lactobacillus</i>	6
	8	<i>Bacillus</i>	5
	9	<i>Bacillus</i>	4
	10	<i>Bacillus</i>	7
	11	<i>Bacillus</i>	11
	12	Gram-positive rods	3
	13	<i>Bacillus</i>	4
3	14	<i>Serratia</i>	4
	15	Gram-positive rods	3
	16	<i>Serratia</i>	4
	17	Gram-positive rods	4
	18	<i>Pseudomonas</i>	4
	19	<i>Xanthomonas</i>	3
	20	heterogeneous cluster*	3
	21	heterogeneous cluster*	3
	22	<i>Pseudomonas</i>	3
	23	Gram-negative rods	3
	24	heterogeneous cluster*	3
	25	<i>Enterobacteriaceae</i>	4
	26	<i>Klebsiella</i>	3
4	27	<i>Xanthomonas</i>	3
	28	<i>Pseudomonas</i>	4
	29	<i>Pseudomonas</i>	3
	30	<i>Pseudomonas</i>	5
	31	Gram-negative rods	4
	32	<i>Pseudomonas</i>	5
	33	<i>Pseudomonas</i>	5
	34	<i>Pseudomonas</i>	10
	35	<i>Pseudomonas</i>	4
	36	<i>Pseudomonas</i>	9
	37	<i>Pseudomonas</i>	3
	38	Gram-negative rods	3
	39	Gram-negative rods	8
	40	<i>Pseudomonas</i>	6
	41	<i>Pseudomonas</i>	9
	42	Gram-negative rods	5
	43	<i>Pseudomonas</i>	13
	44	<i>Pseudomonas</i>	6

* Cluster including more than one genus

The bacterial diversity was greater in the litter than in the phylloplane (fig. 2). Indeed, 26% of the population belonged to other genera than *Bacillus* and *Pseudomonas*. *Lactobacillus* was found on both phylloplane and litter. Lactic acid bacteria such as *L. plantarum* have been previously reported on the olive phylloplane (Ercolani, 1991). Pigmented strains belonging to *Xanthomonas* and *Micrococcus*, that were often found on aerial leaf surfaces (Stout, 1961), were isolated from evergreen oak leaves with weak

percentages (10% of the phylloplane population and less than 4% of the litter population).

Seasonal succession of bacteria on the leaves

Bacterial communities associated to the phylloplane or the litter were dominated by either one or the other of only two genera, *i.e.* (i) *Bacillus* in autumn on 1-year-old leaves, in spring on 2-year-old and L_2 leaves, in summer on L_1 leaves, and (ii) *Pseudomonas* in spring on L_1 leaves, in summer on L_2 leaves, in autumn and winter on both upper layer and underlayer litter (*fig. 2*). *Bacillus* strains were generally more numerous in spring

and summer, *Pseudomonas* in autumn and winter. This tendency was also detectable in the distribution of isolates according to the phenotypic clustering (*fig. 1*). Groups 1 and 2 contained predominantly isolates from spring (42% of the population) and from summer (36%) while groups 3 and 4 contained a large majority of isolates from autumn (38 and 33% respectively). *Bacillus* and *Pseudomonas* were present throughout the year on the litter. This result suggests that leaf surfaces were colonized by several species. *Pseudomonas* population was not abundant in spring on L_2 litter in contrast with L_1 . In summer, *Pseudomonas* increased on L_2 as compared to L_1 . The upper layer litter could protect from climatic variations bacteria present in underlayer litter.

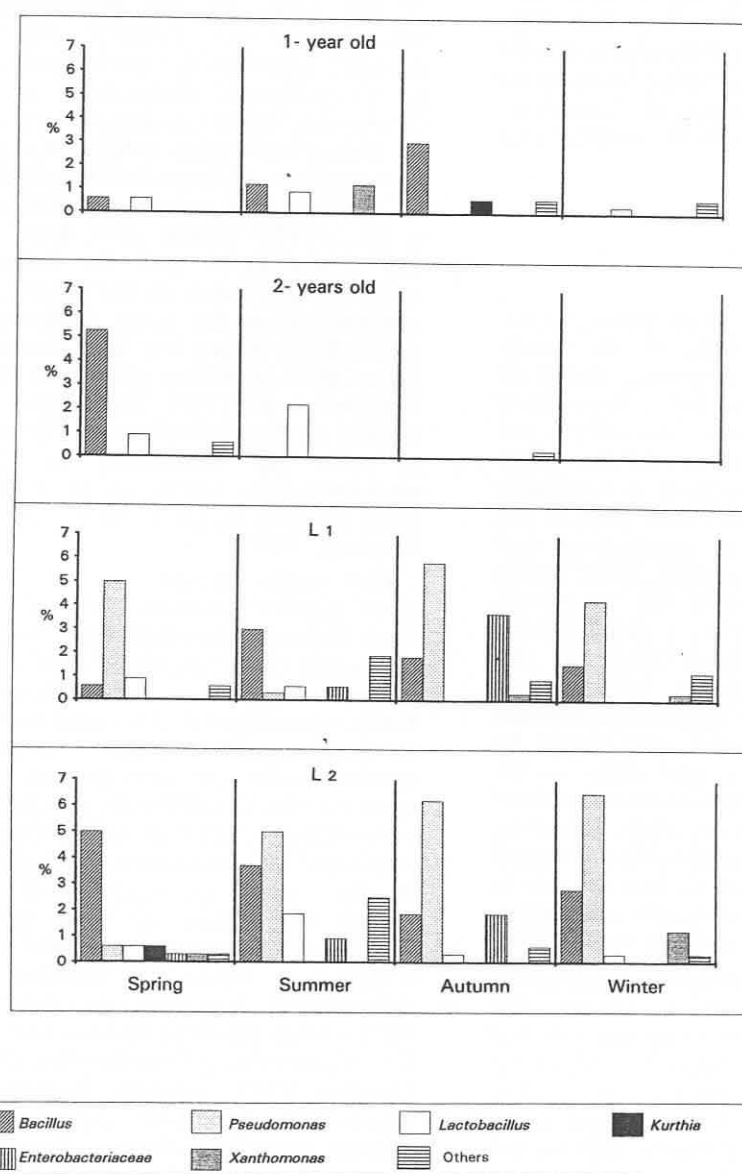


Figure 2. – Taxonomic distribution of the isolates on the various stages of leaves (1-year-old and 2-year-old on the phylloplane, L_1 = litter upper layer, L_2 = litter underlayer) and throughout the seasons. Note: The percentages are expressed with regard to the total number of isolated strains.

The study of bacterial growth at various temperatures showed that the highest specific growth rates of *Bacillus* (0.95 h^{-1}) and *Pseudomonas* (0.76 h^{-1}) were measured at 28°C . Indeed, bacteria that colonize aerial parts of plants or litter leaves are known to be mesophilic bacteria. At 5°C , the mean values of specific growth rates ranged from $0.042 \pm 0.003 \text{ h}^{-1}$ for *Bacillus* strains to $0.120 \pm 0.001 \text{ h}^{-1}$ for *Pseudomonas* strains. At 40°C , the *Bacillus* specific growth rates were higher than those of *Pseudomonas*, particularly in summer ($0.510 \pm 0.2 \text{ h}^{-1}$ for *Bacillus* and only $0.049 \pm 0.029 \text{ h}^{-1}$ for *Pseudomonas* strains). Temperature was therefore one of the climate-related factors that explained the distribution of *Bacillus* and *Pseudomonas* strains on leaves.

Xanthomonas, present mainly in winter, was replaced in spring by *Lactobacillus* that also predominated in summer (fig. 2). *Enterobacteriaceae* including mainly *Klebsiella* and *Serratia* succeeded to *Lactobacillus* in autumn. Percentages of *Enterobacteriaceae* on the litter were weak in summer and increased in autumn (fig. 2).

DISCUSSION

In order to recover bacteria from leaves, some researchers used vigorous shaking of the leaves before plating dilutions of the suspension liquid on a nutrient agar medium (Ercolani, 1978; Donegan *et al.*, 1991; Smith and Couche, 1991). We did not use these dilution plate technique because of the firmly attached bacteria that could constitute a frequently ignored part of the microflora of leaves (Preece and Wong, 1981). Indeed, the abaxial evergreen oak leaf is covered with tufted hairs (Madjidieh, 1986; Racon *et al.*, 1988) that increase greatly the attachment area for bacteria and can serve as a major habitat for survival of resident populations (Schneider and Grogan, 1977; Leben and Whitmoyer, 1979). In our leaf plating method, microscopic observations showed that tufted hairs were not removed from leaves by shaking. The method of plating leaf disks on TS medium underestimates populations because the slow-growers are thus excluded from the study. However, bacteria belonging to the dominant microflora attached on tufted hairs or on other parts of the leaf are able to grow. Attached bacteria are metabolically more active than unattached bacteria (van Loosdrecht *et al.*, 1990).

The cluster analysis showed different communities according to the age of leaves and to seasons. The heterotrophic bacteria attached to evergreen oak leaf surfaces of various ages and at various periods of the year were dominated by *Pseudomonas* and *Bacillus* populations. The abundance of these two genera was previously reported for other ecosystems (Hissett and Gray, 1973; Gyllenberg and Eklund, 1974; Arpin *et al.*, 1986) and could be explained in terms of their opportunistic behaviour on leaf surfaces. The

communities did not show the same metabolism. Some of them produced extracellular hydrolytic enzymes and were able to degrade structural or reserve polymers of the leaves. For example, many populations of *Bacillus* associated to the phylloplane, particularly to the 2-year-old leaves were able to hydrolyze pectic polymers. Moreover, four *Bacillus* strains including two phylloplane strains utilized aromatic lignin monomers. Thus, they may be able to occur on the lignin degradation after the action of fungi on this polymer (Zimmermann, 1990). The role of these strains in the degradation of leaf components requires further study.

The communities present on living leaves were composed of few strains and fewer taxa than those present on dead leaves at the same time of the year. So, the phylloplane of evergreen oak was a very selective habitat for bacterial growth. Unfavorable environmental factors such as microclimatic fluctuations or hydrous stress (Dickinson, 1982) can explain this result. Moreover, the hydrophobic waxy cuticle on leaf surface prevent bacteria from sticking. Antimicrobial substances such as tannins were present on leaf surfaces (Zemek *et al.*, 1987). Tannin cells were observed within the sclerenchyma and the lower epidermis of evergreen oak leaves on the canopy, but were mostly concentrated in the upper epidermis (Racon *et al.*, 1988). Litter leaves lose their tannin compounds and hence their inhibitory effects on bacteria (Poinot-Balaguer *et al.*, 1993). The absence of *Pseudomonas* isolates on the phylloplane may be due to antagonism to other leaf inhabitants. In other ecosystems, phytopathogens belonging to *P. fluorescens* group were reported on green leaves (Austin *et al.*, 1978; Ercolani, 1991).

Our results indicate clearly that, despite their close physical proximity, the phylloplane and the litter bacterial microflora have their own distinctive taxonomic characteristics. Our results therefore agree with the conclusions of Goodfellow *et al.* (1976). Further investigation is required to determine whether the isolates of *Bacillus* present on the phylloplane can survive on the litter after the leaf fall. Superimposed upon the spatial distribution is a seasonal pattern. The composition of the two main bacterial populations is influenced by temperature which is probably involved in the process of selection of the communities specifically adapted to their habitats. Thus, the phylloplane is exposed to great climatic variations which may favour the *Bacillus* growth at high temperatures. A seasonal succession is also observed for the other genera or families found on leaf surfaces, *i.e.* *Xanthomonas*, *Lactobacillus*, *Enterobacteriaceae*. Dominance of *Lactobacillus* in summer was previously reported on the beech leaf by Jensen (1971). In autumn, intensive activity of the soil fauna, including macroarthropods, microarthropods and earth-worms, the faeces of which contain many enterobacteria (Ineson and Anderson, 1985) can explain the

dominance of *Enterobacteriaceae*. We can suppose also that temperature plays a part in this succession.

It will be of interest to study the role of other environmental factors (moisture, sunlight, colonization of the leaf surface by other microorganisms) and of host plant origin on population dynamics.

Acknowledgements

We are grateful to Dr A. Bianchi and Dr R. Matheron for helpful discussions. We are also indebted to J. Garcin for technical advices and to Dr F. Van Wambecke for assistance with computer operation.

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